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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

FEB 4 1974

Application for Research Grant

(Use extra pages as needed)

Date: 1-30-74

1. Principal Investigator (give title and degrees):

Ronald E. Rasmussen, Ph.D. , Assistant Research Physiologist, Cancer Research Institute.

2. Institution & address:

University of California, 3rd Ave. at Parnassus, San Francisco, CA., 94143.

3. Department(s) where research will be done or collaboration provided:

Cancer Research Institute

4. Short title of study:

Effect of Cocarcinogens and Tumor Promotors on DNA Repair in Mammalian Cells Susceptible to Chemical Transformation.

5. Proposed starting date: April 1, 1974

6. Estimated time to complete: 2 years

7. Brief description of specific research aims:

Cell cultures derived from NIH-Swiss mouse embryos will be given a sublethal dose of ultraviolet (UV) light which will produce a known amount of DNA damage and result in a readily measurable amount of DNA repair. Before, after, or simultaneously with the UV exposure, the cells will also be exposed to specific carcinogens, cocarcinogens, or tumor promotors, and the amount of DNA repair (as indicated by incorporation of radioactive precursors into DNA under the conditions described below) will be compared to control cultures not so exposed. The results will bear on the question of whether the compounds tested exert their effects through interfering with DNA repair, and which compounds may be particularly important in this regard. Concurrently, the levels of hydroxylating enzymes (AHH) in the cell cultures and the toxic effects of the test compounds in terms of inhibition of cell proliferation will be measured.

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8. Brief statement of working hypothesis:

DNA is the repository of genetic information in mammalian cells and, as such, its integrity must be maintained. This is thought to be accomplished, in part, by a DNA repair system that is capable of recognizing damaged or distorted regions of DNA, excising them, and restoring the removed region with the proper sequence of nucleotides. It is known that many carcinogenic chemicals bind to DNA both in vivo and in vitro, and further that often this binding stimulates the DNA repair system. Evidence that DNA repair may be closely related to human cancer has been provided by studies of the hereditary disease xeroderma pigmentosum, which is characterized by extreme sensitivity to UV light (e.g. sunlight) and the subsequent development of skin cancer. The DNA repair system has been shown to be defective in individuals with this disease.

Given the relationship among DNA damage, DNA repair, and carcinogenesis suggested by the above findings, it is of interest to determine whether cocarcinogens and tumor promotors may act by interfering with DNA repair. The results of these studies will provide basic information regarding the action of carcinogenic substances, and may indicate the mode of action of the modifiers of carcinogenic activity.

9. Details of experimental design and procedures (append extra pages as necessary)

INTRODUCTION AND BACKGROUND

Repair of Damage to DNA. Damage to the genome (DNA) of mammalian cells or bacteria, whether caused by radiation or certain chemicals, results in the initiation of a special kind of DNA synthesis that has been equated with the repair of the lesion. This process has been called "uncheduled DNA synthesis", "radiation-stimulated DNA synthesis", and "repair replication". In the following discussion the term repair replication will be used.

Repair replication was first studied in bacteria where it was shown by Pettijohn and Hanawalt (1) that the process probably involved a partial degradation of the damaged DNA followed by a resynthesis of the degraded portion. This interpretation was subsequently confirmed by other workers who also showed that, in the case of UV damage, the photoproducts formed in DNA were released from the molecule concomitant with repair replication (2,3). The biological significance of repair replication in bacteria is supported by studies of mutant strains which have been found to lack one or more of the enzymatic activities associated with the process. These strains are characterized by an extreme sensitivity to radiation (4).

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Biochemical evidence that a process similar to the bacterial repair replication was also present in mammalian cells was first reported by Rasmussen and Painter (5,6). Mammalian cells normally double their DNA content in preparation for division during a specific period in their growth cycle (S-phase). However, after exposure to UV light, x-rays, or certain chemicals, mammalian cells will incorporate exogenous DNA precursors into macromolecular DNA whether they are in the S-phase or not. This can be demonstrated by exposing a cell culture to the damaging agent and then labeling the cells with a radioactive DNA precursor such as ^{3}H-thymidine (^{3}H-dT) for a short period. Radioautographs of untreated cells thus labeled will show only a fraction of the cells with silver grains over the nuclei, representing incorporation of the label during normal DNA synthesis. Radioautographs of the treated cells will show incorporation of the label into all cell nuclei, including cells in mitosis. Experiments patterned on those performed with bacteria showed that this special DNA synthesis is different from the normal, semiconservative DNA synthesis which doubles the DNA content of the cell prior to division. As in the bacterial system, the evidence indicates that exogenous DNA precursors are incorporated into relatively short, single-stranded regions of the DNA double helix (6).

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Since the first reports of repair replication in mammalian cells, the phenomenon has been studied in a number of laboratories. The results of these studies have indicated that mammalian cells possess a repair system that is capable of recognizing a number of different kinds of damage and, presumably, attempting repair. The hypothetical repair system consists of at least three parts: An endonuclease which recognizes a defect in DNA, breaks the DNA chain and excises the damaged region; A DNA polymerase which replaces the excised region with the proper sequence, presumably using the opposite strand as a template; A ligase or sealase which makes the final phosphodiester link between the newly-inserted nucleotides and the end of the previously existing DNA strand (6,7,8). The net result is a "cut-and-patch" repair. On the molecular level, we have shown that once the DNA has been "repaired" it can then replicate in a semi-conservative manner as required for normal DNA duplication (9).

Whether or not repair replication in mammalian cells is biologically significant has not been unequivocally proven. However, there is strong circumstantial evidence to support this contention. In humans, the lack of the complete repair replication system is associated with extreme sensitivity to UV light and a predisposition to skin cancer in those individuals exposed to sunlight. Persons with the hereditary disease xeroderma pigmentosum must be protected from sunlight or they develop skin lesions and in many cases skin cancer. In cultures of skin cells from these people, DNA repair replication is absent or defective (10). This finding has been confirmed in vivo (11).

Chemicals that Affect DNA Repair. In attempts to establish the nature of DNA repair replication, a number of inhibitors of DNA synthesis have been tested for their effect on DNA repair. The general findings have been that some compounds inhibit both normal and repair replication, while others inhibit only normal replication (12,13,14). There is no evidence known to this writer for the specific inhibition of DNA repair by any chemical agent. However, since only a relatively small number of compounds have been tested, it is premature to draw any general conclusions. It has been recently reported that several compounds inhibit DNA repair synthesis in unstimulated human lymphocytes (15,16). In this system, DNA repair synthesis is apparently possible, but normal DNA synthesis only occurs after the cells have been treated with a mitogen such as phytohemaglutinin. Hence the effect of the test materials on normal DNA synthesis was not measured. Nevertheless, it is of interest that the cocarcinogens phorbol ester and anthralin inhibited repair replication (16). Therefore, it is important that the effects of these compounds be studied in a cell system that is susceptible to the effects of chemical carcinogens.

Aryl Hydrocarbon Hydroxylases. The mixed-function oxidases or aryl hydrocarbon hydroxylases (AHH) are induced in mammals by a wide variety of chemicals including therapeutic drugs, steroid hormones, and carcinogens (17,18). They are especially important in regard to chemical carcinogenesis because it is generally believed that this group of enzymes is responsible for the activation or conversion of many carcinogens to the ultimate carcinogenic form that then reacts with cellular material to cause malignant transformation (19,20). In our laboratory we have studied the action of the AHH enzymes on the carcinogen benzo[a]pyrene (BP) in cell cultures, organ cultures, and in cell-free preparations from a variety of tissues (21,22,23). We have found that the specific metabolism of BP is characteristic of the species and tissue, and also may be modified by treatment of the animal or cell cultures with various compounds (23,24,25). Of particular interest for the proposed studies, we have found that at least two metabolites of BP, the 4,5-epoxide and another as yet unidentified form, can react in vitro with DNA to give rise to an apparent covalent linkage (22,25). Others have presented similar findings with a variety of different carcinogens that are activated by the AHH enzymes (26,27,28,29). Therefore, it is clear that the AHH enzymes are responsible for the binding of many carcinogens to DNA, and consequently may affect DNA repair. For this reason we feel that it is important to examine the effects of the compounds to be tested on the AHH enzymes, both in terms of induction of enzymatic activity and also the specificity of the enzymatic reactions.

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METHODS OF PROCEDURE

Compounds to be Tested for Their Effect on DNA Repair. Because of its potent action as a cocarcinogen and tumor promotor, the first compound to be tested will be phorbol ester. As noted above, it has been reported as an inhibitor of DNA repair synthesis in human lymphocytes (16). Additional test compounds will include representatives of the materials present in tobacco smoke or as environmental pollutants from other sources. Examples of toxic materials would be CN^-, CO, acetaldehyde, and acrolein. Carcinogenic and noncarcinogenic hydrocarbons are known to physically associate with DNA and thus deserve testing . Other radiation-sensitizing drugs (chloroquine, quinacrine) which have been reported to inhibit DNA repair in human lymphocytes (15) should also be tested in the proposed experiments. It is hoped that, within a few months, fractions of tobacco smoke condensates will become available for testing. However, until that time, there are many individual components that are available as off-the-shelf chemicals.

Cell Type to be Used. NIH-Swiss mouse embryo cells will be used in the first series of experiments. It is planned to obtain the cells as primary cultures from Microbiological Associates, and to use them in the experiments at the first or second passage. In this way we hope to minimize variability in the experimental cultures, and to avoid possible changes that may occur with repeated passage in culture. The cells will be grown in disposable plastic flasks and dishes using Eagle's minimum essential medium supplemented with fetal calf serum.

Depending upon the results with the mouse cells, the experiments may be extended to include Syrian hamster embryo cells, which are also susceptible to in vitro transformation by chemicals.

Cell Toxicity Studies. In order to carry out experiments on the effects of particular compounds on DNA repair it will be necessary to determine the highest concentration of test compounds that will still allow cell survival, so that there will be the greatest chance for detection of possible effects on DNA repair. Furthermore, in order to determine whether the effect of a particular compound is specific for DNA repair or also affects normal DNA synthesis, it will be necessary to know the effects of the test materials on normal DNA synthesis. Therefore, the toxicity studies will measure 1) the rate of cell proliferation as indicated by the cell doubling time and 2) the rate of DNA synthesis as measured by the incorporation of ^{3}H-dT into macromolecular DNA when the cultures are exposed to the test compounds.

Cell proliferation studies will be done by seeding the cells in 60 mm plastic dishes at a concentration (approx. 2-4 x 10^5 cells per dish) that will allow for 3-4 cell doublings before the cell layer becomes confluent. The test compounds will be added to the cell growth medium at various concentrations, and, at intervals, the number of cells in sample dishes will be determined by hemcytometer count. One or two hours prior to harvesting the sample cultures, ^{3}H-dT will be added to the culture dishes in order to label the DNA. Upon harvesting the cells, a portion of them will be processed to determine the extent of incorporation of ^{3}H-dT into macromolecular DNA. This will be done in a manner similar to that described by Scott, et al.(30). Briefly, the method involves digestion of the cells with 1 N NaOH which hydrolyses the RNA, precipitation of the nucleoprtein by the addition of HCl, and treatment of the washed precipitate with hot perchloric acid which hydrolyses the DNA. Measurement of the absorbance of the perchloric acid extract at 265 nm, and determination of the radioactivity in a liquid scintillation spectrometer will indicate the specific radioactivity of the DNA. The latter will then be an index of the rate of DNA synthesis in the treated cells.

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Parallel cultures containing glass coverslips on which the cells will have grown will also be labeled with ^{3}H-dT. At the end of the labeling period, the coverslips will be processed for radioautography. This will allow measurements of the number of cells in S-phase, and, together with the specific radioactivity measurements, will enable the determination of the absolute rate of DNA synthesis, i. e., the rate per cell in S-phase. When compared with the appropriate controls, these experiments will show the effect of the test compounds on normal DNA synthesis. If the test compounds themselves induce DNA repair, this will be apparent in the radioautographs (see below).

DNA Repair Studies. In the proposed experiments the DNA repair process will be stimulated in the cell cultures by exposure to UV light at 245 nm. This choice was made because UV light at this wavelength produces a specific kind of damage to DNA (pyrimidine dimers), and the subsequent DNA repair can be readily quantified.

DNA repair replication can be assayed in two ways. Radioautographic techniques can be used to demonstrate repair replication in cell cultures after exposure to radiation or chemicals. In this method, the cell cultures are labeled with high specific activity ^{3}H-dT after treatment with the damaging agent. Radioautographs are prepared, and after exposure and development, repair replication can be quantified by counting the silver grains in the emulsion overlaying the cell nuclei (6). Cells participating in repair replication can be distinguished from those in normal DNA synthesis at the time of labeling because the rate of repair replication is of the order of 1% of the rate of normal DNA synthesis. Therefore, by proper choice of labeling time and exposure time, cell nuclei undergoing repair replication will appear lightly labeled with a countable number of silver grains while those in normal DNA synthesis will appear completely covered.

An alternate method of measuring DNA repair involves the use of CsCl gradient techniques and high specific activity ^{3}H-bromodeoxyuridine (^{3}H-BrdU), an analog of thymidine. During normal DNA synthesis, ^{3}H-BrdU is incorporated into new DNA strands in place of thymidine and, because of the presence of the bromin atom, the new DNA has a higher buoyant density than normal.(32). By centrifuging the isolated DNA in a concentrated solution of CsCl, new DNA can be separated from that which did not replicate during the period of labeling with ^{3}H-BrdU. During centrifugation, a concentration gradient of CsCl forms in the centrifuge tube, and the individual DNA molecules will come to equilibrium in this gradient at a position where their buoyant density equals that of the CsCl solution. Nearly all of the radioactivity is found associated with the newly made DNA of high buoyant density. As discussed above, repair replication appears to involve the degradation and resynthesis of relatively short, single-stranded regions of DNA. When ^{3}H-BrdU is used as a label following treatments which damage DNA in cell cultures, and the DNA is extracted and analysed as described, radioactivity is found associated with both the newly-made DNA and also with the unreplicated DNA (6,9,33). The conclusion is that ^{3}H-BrdU is incorporated into DNA during repair replication, but the amount incorporated is too small to significantly change the buoyant density of the isolated DNA molecules. Therefore, using this method, one can assay repair replication by measuring the specific radioactivity of unreplicated DNA that has undergone repair replication.

Both of the assay methods described would be used in the proposed studies.

AHH Studies. In the proposed experiments the AHH enzymes in the cell cultures would be assayed using the methods that we have developed in conjunction with other studies in this laboratory. These techniques were adapted from those described earlier by Nebert and Gelboin (34,35), and Grover and Sims (36). Briefly, the procedure involves exposure of the cell cultures to the test compound for several hours, followed by the preparation of microsomal fraction or homogenate of the cells. The cell-free preparation then serves as the source of AHH activity, which is assayed under standardized conditions. In the proposed studies, ^{3}H-benzo[a]pyrene would be used as

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the substrate for the AHH enzymes, and analysis of the products would be done using thin-layer chromatography. This would allow the quantification of metabolic products, and also the detection of possible changes in the spectrum of products as a result of the experimental treatment. The details of this procedure will be found in the accompanying reprints (Wang, et al., BBRC 49:1142, 1972., Borgen, et al., J. Med. Chem. 16: 502, 1973).

Estimated Experimental Schedule. The estimated time for completion of the studies for each of the compound to be tested is 6-8 weeks. However, since some of this time represents the exposure time for the radioautographs, it will be possible to work with several (i. e. 4-6) compounds simultaneously. Therefore, in the first year we can expect to have data on about 40 compounds.

References

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Laboratory space of approximately 600 ft^2 is now assigned to the applicant for use in these and related studies. The laboratory is equipped with a fume hood for use with volatile or toxic materials.

Major Equipment:

Packard TriCarb liquid scintillation spectrometer
Beckman-Spinco Model L preparative ultracentrifuge
NAPCO CO_2 Incubator
Beckman Model DU Spectrophotometer

Other facilities available on a shared basis include a darkroom and walk-in cold room.

11. Additional facilities required:

None

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Source: https://www.industrydocuments.ucsf.edu/docs/nldm0000

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Rasmussen, Ronald E., Ph.D.	50%	$10505
Technical		
Staff Research Associate I (to be recruited)	100%	$10971
Sub-Total for A		$21476

B. Consumable supplies (by major categories)	
Radioactive Compounds	$ 1600
Disposable plastic ware	1000
Glassware	300
Photographic emulsion for radioautography	300
Cell cultures and cell culture medium	2600
Chemicals, including test cmpds., CsCl, NADPH, etc.	1000
Sub-Total for B	$ 6800

C. Other expenses (itemize)	
Radioactive waste disposal	$ 150
Equipment maintenance contracts	300
Estimated equipment repairs	200
Medical illustration, Publications	500
Travel for R. E. Rasmussen	500
Misc., office supplies, etc.	200
Sub-Total for C	$ 1850
Running Total of A + B + C	$30126

D. Permanent equipment (itemize)

None

Sub-Total for D	-0-
E	$ 4519
Total request	$34645

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$23000	$7000	$2000	-0-	$4800	$36800
Year 3						

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Chemical carcinogenesis and mammalian DNA replication.	U.S.P.H.S. CA-11939-03	$36,768	6/1/73-5/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Metabolism of Carcinogens in animals and humans	U.S.P.H.S. CA-11939-04	$56,068	6/1/74-5/31/75
Environmental pollution and carcinogen metabolism	U.S.P.H.S. ES-CA-01070	$34,359	7/1/74-6/30/75

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Ronald E. Rasmussen, Ph.D.

Signature Ronald E. Rasmussen Date 1/28/74

Telephone 415 (Area Code) 666-1041 (Number) (Extension)

Checks payable to

Regents of the University of California

Mailing address for checks

Gifts & Endowments Office
University of California
San Francisco, California 94143

Responsible officer of institution

Typed Sue Clark
Program Coordinator

Title Gifts and Endowments

Signature Sue Clark Date 1/31/74

Telephone 415 (Area Code) 666-2047 (Number) (Extension)

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